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A comprehensive model of purine uptake by the malaria parasite *Plasmodium falciparum*: identification of four purine transport activities in intraerythrocytic parasites

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Plasmodium falciparum is incapable of *de novo* purine biosynthesis, and is absolutely dependent on transporters to salvage purines from the environment. Only one low-affinity adenosine transporter has been characterized to date. In the present study we report a comprehensive study of purine nucleobase and nucleoside transport by intraerythrocytic *P. falciparum* parasites. Isolated trophozoites expressed (i) a high-affinity hypoxanthine transporter with a secondary capacity for purine nucleosides, (ii) a separate high-affinity transporter for adenine, (iii) a low-affinity adenosine transporter, and (iv) a low-affinity/high-capacity adenine carrier. Hypoxanthine was taken up with 12-fold higher efficiency than adenosine. Using a parasite clone with a disrupted *PfNT1* (*P. falciparum* nucleoside transporter 1) gene we found that the high-affinity hypoxanthine/nucleoside transport activity was completely abolished, whereas the low-affinity adenosine transport activity was unchanged. Adenine transport was increased, presumably to partly compensate for the loss of the high-affinity hypoxanthine transporter. We thus propose a model for purine salvage in *P. falciparum*, based on the highly efficient uptake of hypoxanthine by PfNT1 and a high capacity for purine nucleoside uptake by a lower affinity carrier.

Key words: drug target, equilibrative nucleoside transporter family, nucleobase transporter, PfNT1, *Plasmodium falciparum*, purine salvage.

INTRODUCTION

Plasmodium spp. are auxotrophic for purines, as are all other parasitic protozoa studied to date, and require an efficient salvage system for these essential nutrients [1]. Purine salvage may be especially important during the fast dividing intraerythrocytic stages, and it has long been known that *Plasmodium* trophozoites accumulate some purines very rapidly [1,2]. Tracy and Sherman [2] found that hypoxanthine, adenosine and inosine, but not adenine or nucleotides, were efficiently taken up by the avian malaria parasite *P. lophurae*. Similarly, the rodent malaria *P. berghei* was reported to have two separate uptake systems for purines: one for adenine and one for adenosine, inosine and hypoxanthine [3]. The study of purine metabolism in the human malaria species *P. falciparum*, however, showed clearly that hypoxanthine was by far the preferred purine source for the parasite, with very high activities of hypoxanthine–guanine phosphoribosyltransferase and inosine phosphorylase but not of adenine phosphoribosyltransferase or adenosine kinase [4]. Adenosine salvage appeared to proceed through rapid deamination to inosine and thence to hypoxanthine [4], and this process mostly occurs extracellularly [5]. This explains the observation of Hansen et al. [3] that inhibition of adenosine deaminase with deoxycoformycin potently reduced [^{3}H]adenosine uptake by *P. berghei*. The resulting model of a crucial role for hypoxanthine transport in *Plasmodium* purine salvage was further validated by the study of Berman et al. [6] who showed that depletion of hypoxanthine from infected erythrocytes by xanthine oxidase reduced *P. falciparum* growth by ~90 %.

Although hypoxanthine transport clearly plays a pivotal role in an essential function of the malaria parasite, almost all available information on *Plasmodium* purine transporters concerns uptake of adenosine. Penny et al. [7] injected *Xenopus* oocytes with mRNA from *P. falciparum* and observed increased uptake of adenosine and hypoxanthine, sensitive to at least partial reciprocal inhibition, demonstrating the presence of at least one transporter capable of recognizing both substrates. Two groups have independently cloned the same *P. falciparum* gene of the ENT (equilibrative nucleoside transporter) family, which they termed *PfENT1* (*P. falciparum* ENT1) or *PfNT1* (*P. falciparum* nucleoside transporter 1) respectively [8,9], and the PfNT1 protein was found to be localized in the plasma membrane of the parasite [10]. Both groups expressed the cloned transporter in *Xenopus* oocytes for characterization, but they arrived at strikingly different conclusions. Carter et al. [9] described PfNT1 as a broad-specificity nucleoside transporter of moderately high affinity for adenosine and inosine and no affinity for nucleobases. In contrast, Parker et al. [8] report a purine nucleoside/nucleobase transporter with a similar, but low, affinity for adenosine, adenine and hypoxanthine. The presence of a low-affinity adenosine transporter was recently confirmed by Downie et al. [11] using *P. falciparum* trophozoites isolated after treatment with saponin. On the basis of the adenosine K_m value, they concluded that this activity must be PfNT1, and this seemed to be confirmed

Abbreviations used: ENT, equilibrative nucleoside transporter; hFNT1, human facilitative nucleoside transporter 1; JA-23, 2-amino-*N*^{6}-amino-*N*^{6}-methyladenosine; JA-24, 2-amino-*N*^{6}-amino-adenosine; JA-32, *N*^{6}-hydroxy-9*H*-purin-6-amine; PfADET, *Plasmodium falciparum* adenine transporter; PfENT1, *Plasmodium falciparum* ENT1; PfLAAT, *Plasmodium falciparum* low-affinity adenosine transporter; PfNT1, *Plasmodium falciparum* nucleoside transporter 1.

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by expression of *PfNT1* in oocytes, which revealed a low-affinity nucleoside transporter. However, in the present study we will demonstrate conclusively that the low-affinity adenosine transporter is not encoded by *PfNT1*.

Studies to date have not investigated the possibility of a very high-affinity purine transporter, such as found in other intracellular [12–14] and extracellular [15,16] protozoan parasites. However, a recent report [17] did strongly suggest that *PfNT1* must encode a high-affinity purine transporter, as wild-type *P. falciparum* were able to grow in very low levels of purines, but Δ*PfNT1* parasites grew only in $>10\ \mu$M hypoxanthine, adenosine or inosine. The same study also showed that, at 1 μM concentrations, accumulation of those three purines was reduced in Δ*PfNT1*, but the characteristics of this transporter in terms of substrate affinity and specificity were not further investigated.

In the present study we report the first systematic study of high-affinity hypoxanthine, adenine and adenosine transport activities in *P. falciparum* trophozoites. We have identified and characterized a broad-specificity carrier that transports hypoxanthine, guanine, adenosine, guanosine and inosine, but not adenine. In addition we characterized a low-affinity adenosine transporter [denoted PfLAAT (*P. falciparum* low-affinity adenosine transporter)] and a high-affinity adenine transporter [denoted PfADET1 (*P. falciparum* adenine transporter 1)] and demonstrate the existence of a further low-affinity uptake route for adenine (PfADET2). To confirm our hypothesis that the high-affinity transport activity is encoded by the previously reported *PfNT1*, we generated a mutant parasite clone with a disrupted *PfNT1* gene, designated Δ*pfnt1*^{GU} to distinguish itself from the clone generated by El Bissati et al. [17]. Δ*pfnt1*^{GU} had completely lost both the high-affinity adenosine-uptake capacity and over 85 % of hypoxanthine uptake, whereas low-affinity adenosine uptake was unchanged and high-affinity adenine uptake was slightly increased. We therefore conclude that *PfNT1* encodes a high-affinity hypoxanthine/purine nucleoside transporter.

In the present study we show for the first time a comprehensive model of purine uptake by *P. falciparum*, in which the intraerthrocytic stages express at least four distinct purine transport activities. This model finally identifies the biochemical and genetic basis for the overwhelming reliance of the parasite on hypoxanthine salvage as its main purine source and is in line with a recent analysis of the *P. falciparum* permeome that showed the presence of four genes of the ENT family in its genome [18]. This family includes all protozoan purine transporters known to date [1].

EXPERIMENTAL

Plasmodium lines, human cells and chemicals

All transport experiments were performed with the standard 3D7 drug-sensitive laboratory clone of *P. falciparum*, originally obtained from David Walliker (School of Biological Sciences, University of Edinburgh, Edinburgh, Scotland, U.K.). Human blood and serum used for *Plasmodium* culture were obtained from the Glasgow and West of Scotland Blood Transfusion Service. Standard chemicals, including purines and pyrimidines, were obtained from Sigma and of the highest purity available. The purine analogues JA-23 (2-amino-N^6-amino-N^6-methyladenosine), JA-24 (2-amino-N^6-amino-adenosine) and JA-32 (N^6-hydroxy-9*H*-purin-6-amine) were donated by Daniel Brown and David Loakes (MRC Laboratory of Molecular Biology, Cambridge, U.K.).

Culturing of *P. falciparum* for transport assays

Asexual parasites of *P. falciparum* were maintained in continuous culture using slightly modified standard methods [19]. Briefly, parasites were grown in RPMI 1640 medium supplemented with 5.94 g/l Hepes, 0.21 % $NaHCO_3$ and 10 % heat-inactivated normal human serum at 5 % haematocrit. The culture was incubated at 37 °C under a gas mixture of 1 % O_2, 3 % CO_2 and 96 % N_2, and medium was changed daily. Parasitaemia of the culture was routinely maintained below 5 %, unless otherwise stated. Prior to use in experiments, parasite cultures were synchronized using a previously described method [20]. Transport experiments were performed with cultures of parasitaemia between 7 and 10 % in order to maximize yield.

Saponin permeabilization of *P. falciparum*-infected erythrocytes

Permeabilized *P. falciparum*-infected erythrocytes were prepared by incubating parasitized red blood cells with 0.15 % (w/v) saponin as described previously [21]. Briefly, the culture was centrifuged at 600 ***g*** and the supernatant was removed. The cell pellets were re-suspended in 5 vol of 0.15 % (w/v) saponin in PBS (137 mM NaCl, 27 mM KCl, 1.76 mM K_2HPO_4 and 8 mM Na_2HPO_4) for 1 min. The freed parasites were washed three times with purine-free RPMI 1640 culture medium without serum, and resuspended in the same medium. Parasitaemia was determined by microscopical examination of thin blood smears stained with Giemsa's stain, and red-cell density was estimated using a Neubauer counting chamber.

Transport assays with saponin-permeabilized *Plasmodium falciparum*-infected erythrocytes

Transport assays were performed with synchronized cultures in the trophozoite stage. After permeabilization of the erythrocytes with saponin [22], which leaves the parasite plasma membrane intact [23,24], the cells were resuspended in purine-free RPMI 1640 at a concentration of 5×10^8 cells/ml. Uptake of [^{3}H]hypoxanthine (31 Ci/mmol; GE Healthcare), [^{3}H]adenosine (16.0 Ci/mmol; GE Healthcare) or [^{3}H]adenine (32.2 Ci/mmol; PerkinElmer) by *P. falciparum* trophozoites was measured using a rapid stop/spin method essentially as described previously for *Trypanosoma brucei* [25], *Leishmania* spp. [14,26], *Toxoplasma gondii* [13] and human erythrocytes [27]. Transport was measured either at various time intervals to generate plots of the uptake rate against time, or over fixed time intervals in the presence of variable inhibitor concentrations to generate plots for the determination of K_m and K_i values, using non-linear regression (GraphPad Prism version 4). Briefly, equal volumes of a suspension of permeabilized-infected erythrocytes and a radiolabelled permeant at twice its final concentration (as well as inhibitor at 2 × concentration, where applicable) were mixed for a predetermined time. The influx of permeant into the cells was terminated by adding 1 ml of ice-cold stop solution (unlabelled permeant at saturating concentrations) and immediately pelleting the cells by centrifugation at 13 000 ***g*** in a microfuge through an oil-mix [300 μl of five parts dibutylphthalate (Aldrich)/four parts dioctylphthalate (Aldrich), v/v], thus preventing further uptake of permeant, which remains in the aqueous layer. Non-mediated influx of the respective permeant was assessed by determining the rate of uptake of the radiolabelled permeant in the presence of a saturating concentration of unlabelled permeant at both room temperature (22 °C) and 0 °C.

The parasite pellets were processed after uptake using the method previously described by Saliba et al. [28], with a slight modification. The aqueous phase was removed by aspiration

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and the inside walls of the tube were carefully washed with distilled water. The water was aspirated out, followed by the oil-mix, and the inside of the tube wiped with folded tissue paper. The cells were resuspended in 200 μl of 1 % (v/v) Triton X-100 in water for 10 min at room temperature, and proteins were precipitated with 200 μl of 5 % TCA (trichloroacetic acid). The mixture was finally centrifuged for 7 min at 13 000 ***g***, and the supernatant transferred into a scintillation vial and mixed with 3.5 ml of scintillation fluid (OptiPhase HiSafe; PerkinElmer). Radioactivity was determined with a PerkinElmer 1450 Microbeta Wallac Trilux liquid scintillation counter. All uptake experiments were performed in triplicate.

For any given permeant, the linear phase of uptake was first determined with a constant permeant concentration incubated for various times to generate a plot of uptake against time. Linearity was assessed using linear regression and defined as a correlation coefficient >0.95 and a significant difference from zero uptake (F-test; GraphPad Prism version 4). When uptake was too rapid to obtain linearity of transport at room temperature, this was reassessed at 6 °C using a ThermoStat plus machine (Eppendorf), as indicated in the Results and discussion section. Inhibition studies were always performed well within the linear phase of uptake, and thus reflect true initial rates of transport across the *P. falciparum* plasma membrane rather than rates of metabolism or sequestration.

Generation of *P. falciparum* clones with disrupted PfNT1

Our strategy for knocking-out the *Plasmodium* gene *PfNT1* relies on single crossover homologous recombination and full details of the methods are provided in the Supplementary data (at http://www.BiochemJ.org/bj/411/bj4110287add.htm). Briefly, a plasmid was constructed containing the central sequence of *PfNT1* spanning the predicted transmembrane domains 3–6 and part of transmembrane domain 2. The construct was used to transfect *P. falciparum* parasites of clone 3D7 using standard methods [29], with control parasites treated similarly but with no construct present during electroporation. Transformed parasites were selected with blasticidin and cloned. Successful disruption of the gene was confirmed using PCR and Southern blot analysis as described in Supplementary data (at http://www.BiochemJ.org/bj/411/bj4110287add.htm).

RESULTS AND DISCUSSION

High-affinity hypoxanthine transport in *P. falciparum* trophozoites

In order to study the transporters in the plasma membrane of *P. falciparum*, infected human erythrocytes were permeabilized with saponin, after which the erythrocyte plasma membrane ceases to be a barrier to the passage of solutes [22] but the parasite membranes are not significantly affected by this saponin treatment protocol [23,24]. The parasitophorous vacuole membrane remains, but this membrane is intrinsically permeable to small solutes [30]. Therefore, initial rates of transport measured in this system reflect uptake by the *Plasmodium*-encoded transporters located in the plasma membrane of the parasite.

Uptake of 0.25 μM or 0.1 μM [^{3}H]hypoxanthine followed a hyperbolical curve, which was linear for up to 120 s at 20 °C, with a rate of 0.62 ± 0.04 and 0.40 ± 0.03 pmol $\cdot$ (10^7 cells)$^{-1} \cdot$ s^{-1} respectively, and was completely inhibited by 1 mM hypoxanthine (Figure 1A). The hypoxanthine thus accumulated would, in the absence of metabolism, reach very high intracellular concentrations: based on an intracellular volume of 2.8×10^{-7} l/10^7 parasites isolated by saponin treatment [28], uptake of 0.25 μM hypoxanthine would be over 1000-fold concentrated in the parasite, reaching a level of 260 μM in 2 min. Transport rates remained constant during this time (i.e. linear phase of uptake) because the rate of metabolism was greater than the rate of transport, making transport the rate-limiting step. Alternatively, high-affinity purine transport in *P. falciparum* may be energy-dependent, as it is in other protozoan species [16,25,31], allowing uptake against a concentration gradient, but this possibility was not further investigated in the present study.

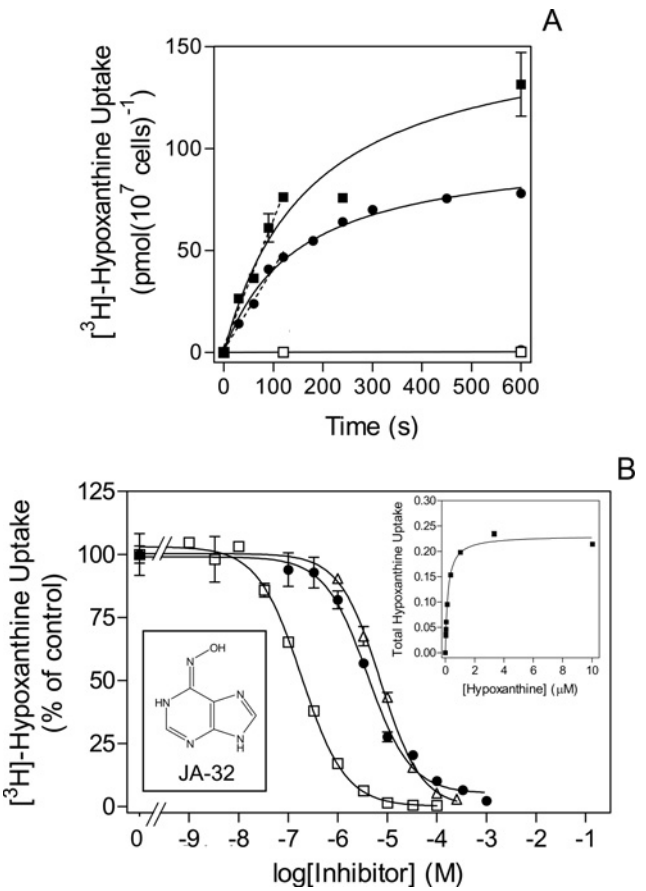

Figure 1 Hypoxanthine transport in saponin-isolated *P. falciparum* trophozoites

(**A**) Uptake of 0.25 μM [^{3}H]hypoxanthine (squares) and 0.10 μM [^{3}H]hypoxanthine (circles) into saponin-permeabilized *P. falciparum*-infected erythrocytes in the presence (open symbols) or absence (filled symbols) of 1 mM unlabelled hypoxanthine at 20 °C. The correlation coefficients (r^2) were determined as 0.99 and 0.98 for 0.25 μM and 0.1 μM radiolabel respectively, by linear regression over the first 120 s. (**B**) Uptake of 30 nM [^{3}H]hypoxanthine in the presence or absence of various concentrations of unlabelled hypoxanthine (□), adenosine (●) or JA32 (△). Uptake rates are presented as the percentage of no-inhibitor controls and combined from two experiments with 100 % values of 0.013 (adenosine and JA32) and 0.033 pmol $\cdot$ (10^7 cells)$^{-1} \cdot$ s^{-1} (hypoxanthine) respectively. Inset: conversion of hypoxanthine inhibition data into a Michaelis–Menten plot for determination of K_m and V_{max}. Representative experiments are shown, conducted in triplicate. Values are means $\pm$ S.E.M.

Determining [^{3}H]hypoxanthine transport at sub-micromolar concentrations revealed a very high-affinity transporter for oxopurines, with a mean K_m value for hypoxanthine of 0.34 ± 0.05 μM ($n = 6$; Figure 1B, inset), a V_{max} of 0.36 ± 0.12 pmol $\cdot$ (10^7 cells)$^{-1} \cdot$ s^{-1} ($n = 6$) and a mean K_i value of 0.11 ± 0.01 μM for guanine ($n = 3$). In addition, the nucleosides adenosine (Figure 1B), inosine and guanosine displayed affinity in the low micromolar range (Table 1). This hypoxanthine transporter with a high affinity for purine nucleosides was designated PfNT1 based

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Table 1 Profile of purine transport in *P. falciparum* trophozoites

Values in bold are K_m values obtained with radiolabelled substrate; other values are K_i values obtained from dose-dependent inhibition of radiolabelled substrate. All values were determined using non-linear regression from experiments in duplicate or triplicate with a minimum of eight points over the relevant range. Zero values were taken to be radiolabel associated with the cell pellet in the presence of saturating concentrations of unlabelled permeant. [a]Units for V_{max} are pmol · $(10^7$ cells$)^{-1}$ · s^{-1}. ND, not determined.

	High-affinity [^{3}H]-hypoxanthine uptake (PfNT1)		High-affinity [^{3}H]-adenosine uptake (PfNT1)		Low-affinity [^{3}H]-adenosine uptake (PfLAAT)		High-affinity [^{3}H]-adenine uptake (PfADET1)	
	K_i or K_m value (at 22 °C; μM, ± S.E.M.)	*n*	K_i or K_m value (at 22 °C; μM, ± S.E.M.)	*n*	K_i or K_m value (at 6 °C; μM, ± S.E.M.)	*n*	K_i or K_m value (at 22 °C; μM, ± S.E.M.)	*n*
V_{max}[a]	0.36 ± 0.12	6	0.18 ± 0.08	3	0.19 ± 0.03	3	0.0004 ± 0.0002	3
V_{max}/K_m	1.1		0.090		0.00096		0.0019	
Hypoxanthine	**0.34 ± 0.05**	6	0.75 ± 0.18	3	ND		>1000	3
Guanine	0.11 ± 0.01	3	ND		ND		>50	3
Inosine	2.0 ± 0.2	3	ND		ND		>1000	3
Adenosine	4.0 ± 0.67	3	**2.0 ± 0.2**	3	**197 ± 20**	3	2.0 ± 0.2	3
Guanosine	11.6 ± 2.7	3	ND		ND		ND	
Adenine	> 500	3	240 ± 70	3	ND		**0.23 ± 0.07**	3

on our later observations linking the *PfNT1* gene to this transport activity (see below).

PfNT1 exhibited a low affinity for pyrimidines. Only uridine, uracil and thymidine inhibited transport of 30 nM [^{3}H]hypoxanthine significantly at a concentration of 1 mM inhibitor, by 36 ± 9 % ($P < 0.05$), 49 ± 6 % ($P < 0.01$) and 57 ± 6 % ($P < 0.02$) respectively (measured using a paired Student's *t* test against a no-inhibitor control; based on four independent experiments in duplicate). Cytosine, thymine and cytidine (up to 1 mM) did not significantly inhibit 30 nM [^{3}H]hypoxanthine transport (results not shown). This result is not surprising, in the light of the well-documented inability of *Plasmodium* spp. to salvage preformed pyrimidine nucleobases and nucleosides [1,32].

Of all of the natural purines tested, only adenine had very little effect on transport of 30 nM [^{3}H]hypoxanthine, with less than 50 % inhibition at 1 mM. However, the antiplasmodial adenine analogue JA-32 [33] displayed a K_i value of 14 ± 3 μM (Figure 1B), probably as a result of its 'hypoxanthine-like' conformation (i.e. lactam hydrogen on N1 and hydrogen-bond acceptor at the N^6 position; see Figure 1B). Two N^6-substituted adenosine analogues, JA-23 and JA-24, with antiplasmodial activities in the low micromolar range [33], had little ($K_i = 320 \pm 80$ μM for JA-24) or no (JA23) effect on high-affinity hypoxanthine transport (results not shown).

The transporter appeared to display a preference for oxopurine nucleobases over aminopurine bases whereas this preference appeared to be curiously absent in the binding of nucleosides: e.g. guanine is the highest affinity nucleobase whereas guanosine is the lowest affinity purine nucleoside (Table 1). Two testable hypotheses may be proposed to explain this apparent paradox: (i) the nucleosides are not actually substrates, but allosteric inhibitors of hypoxanthine transport, or (ii) the nucleosides and nucleobases assume a different orientation within the binding pocket, due to the presence of the ribose moiety. In order to distinguish between these alternatives, we assessed the uptake of low concentrations of [^{3}H]adenosine.

High-affinity adenosine transport in *P. falciparum* trophozoites

Uptake of 0.25 μM [^{3}H]adenosine proceeded with a rate of 0.015 ± 0.002 pmol · $(10^7$ cells$)^{-1}$ · s^{-1} over the linear phase (at least 30 s), and was completely inhibited by 1 mM adenosine (see Supplementary Figure S1 at http://www.BiochemJ.org/bj/411/bj4110287add.htm). At the end of the linear phase, cells had transported approx. 0.55 pmol of [^{3}H]adenosine/10^7 cells, which would correspond to ~8-fold the extracellular concentration if unmetabolized. Both the rate of uptake and the level of concentration of adenosine were much less than for hypoxanthine.

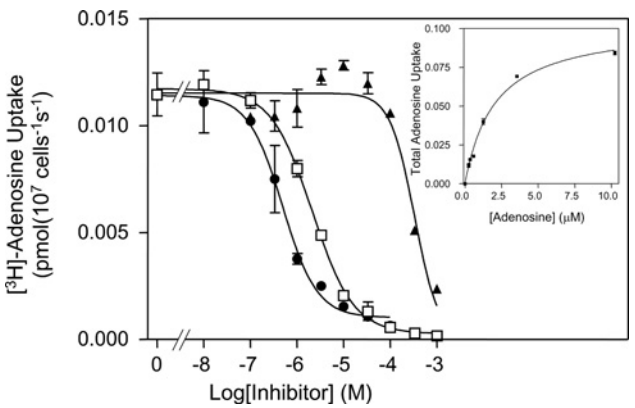

Figure 2 Uptake of low concentrations of [^{3}H]adenosine in isolated *P. falciparum* trophozoites

Transport of 0.25 μM [^{3}H]adenosine was measured over 30 s in the presence or absence of various concentrations of unlabelled adenosine (□), adenine (▲) and hypoxanthine (●). Inset: conversion of the adenosine inhibition data into a Michaelis–Menten plot. The adenine IC_{50} value was obtained by extrapolation to 100 % inhibition. Representative experiments performed in triplicate are shown. Values are means ± S.E.M.

Uptake of 0.25 μM [^{3}H]adenosine was potently inhibited by hypoxanthine (mean $K_i = 0.75 \pm 0.18$ μM; $n = 3$) and unlabelled adenosine, but only by very high concentrations of adenine (Figure 2). Over three separate experiments, the average of the extrapolated values for an adenine IC_{50} value would be ~1 mM, three orders of magnitude lower affinity than for hypoxanthine. In the same three experiments, the inhibition by hypoxanthine was not quite complete at 100 μM (6.1 ± 0.2 %; $P < 0.05$, paired Student's *t* test), possibly the result of a minor contribution to 0.25 μM [^{3}H]adenosine flux from a second, low-affinity adenosine transporter, that is insensitive to hypoxanthine (see below). The mean K_m for adenosine on the high-affinity transporter was determined to be 2.0 ± 0.2 μM and the V_{max} as 0.18 ± 0.08 pmol · $(10^7$ cells$)^{-1}$ · s^{-1} ($n = 3$; Figure 2, inset). The strikingly similar reciprocal K_m and K_i values for adenosine and hypoxanthine uptake are consistent with high-affinity uptake

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of both hypoxanthine and adenosine by PfNT1. However, the V_{max}/K_m ratio indicates an 11.9-fold higher efficiency of translocation for hypoxanthine compared with adenosine, consistent with the preference for hypoxanthine for optimal growth *in vitro*.

The different selectivity for aminopurine nucleobases and nucleosides noted in the previous section thus seems to indicate that nucleobases and nucleosides orient differently in the same binding site and the kinetic profiles shown in the present study are consistent with competitive inhibition (Figures 1B and 2). There are precedents for this; for instance, there is good evidence for different binding orientations for xanthine and uric acid in the *Aspergillus nidulans* UapA purine transporter [34].

Uptake of [^{3}H]adenine is mediated by a separate high-affinity transporter

At 1 μM [^{3}H]adenine, uptake was 0.0091 ± 0.0025 pmol $\cdot$ (10^7 cells)$^{-1} \cdot s^{-1}$ over the linear phase (6 s; $r^2 = 0.93$) and mostly inhibited by 1 mM unlabelled adenine (results not shown). At 120 s the accumulation ratio (intracellular/extracellular radiolabel concentration based on the intracellular volume of 2.8×10^{-7} l/10^7 parasites cited above) had reached ~ 0.6, consistent with an equilibrative process and at best a slow rate of metabolism or no metabolism at all. We tentatively designate this novel transport activity PfADET1, pending the identification of the encoding gene.

In order to determine the K_m value for this high-affinity transporter, uptake of adenine was assessed at 50 nM of radiolabel. Uptake was linear and did not reach equilibrium for up to 240 s (the accumulation ratio was 0.65) (Figure 3A). This allowed inhibition experiments at an incubation time of 150 s, although it was clear that there was a small uptake component that was not sensitive to 1 mM adenine (Figure 3A), possibly resulting from the presence of a very low-affinity transporter. Adenine transport was not inhibited by the oxopurines hypoxanthine, guanine and inosine (results not shown), though it was sensitive to adenosine, with a K_i value of 2.0 ± 0.2 μM (Figure 3B).The sub-micromolar K_m value, similar to the hypoxanthine K_m for PfNT1, and very low V_{max} (Figure 3B), make PfADET1 a high-affinity but low-capacity transport system with a maximum uptake rate 2–3 orders of magnitude lower than that measured for high-affinity transport of hypoxanthine and adenosine (Table 1).

A very low-affinity/high-capacity transporter of adenine

As the uptake of 0.05 μM [^{3}H]adenine was consistently not fully inhibited by 1 mM unlabelled adenine (see above), we investigated the possible presence of a low-affinity adenine transport activity in saponin-isolated *P. falciparum* trophozoites. Uptake of 10 μM [^{3}H]adenine was linear for up to 12 s, with a rate of 0.13 ± 0.01 pmol $\cdot$ (10^7 cells)$^{-1} \cdot s^{-1}$ ($r^2 = 0.96$), reaching an accumulation ratio of 0.85 in 30 s. This could not be entirely attributed to simple diffusion as it was partly inhibited ($\sim 60\%$) by 1 mM adenine (Supplementary Figure S2A at http://www.BiochemJ.org/bj/411/bj4110287add.htm). Attempts to establish a K_m value for [^{3}H]adenine uptake on this transporter were unsuccessful, due in part to the high rate of diffusion of adenine at high concentrations, as reported for the related apicomplexan parasite *Toxoplasma gondii* [13], and because the limitations of adenine solubility prevented the determination of a complete inhibition curve (Supplementary Figure S2B at http://www.BiochemJ.org/bj/411/bj4110287add.htm). However, the K_m for adenine was estimated to be approx. 1 mM, and the K_i value for adenosine >2.5 mM. There was no clear inhibition by up to 1 mM hypoxanthine (results not shown). This observation,

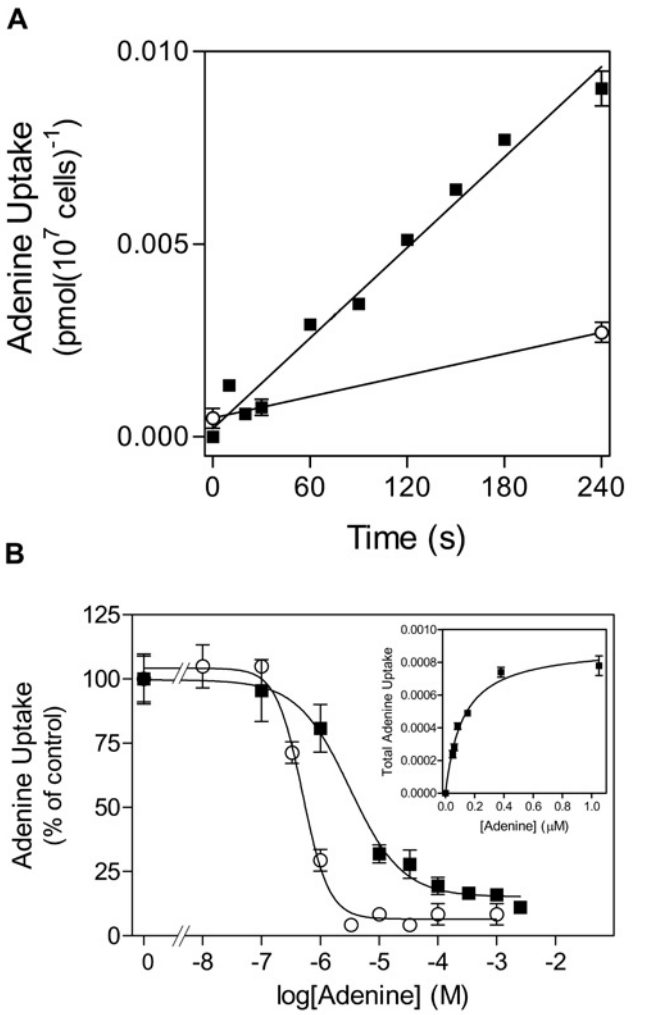

Figure 3 Characterization of a high-affinity adenine transporter in *P. falciparum* trophozoites

(**A**) Transport of 0.05 μM [^{3}H]adenine over 240 s, in the presence (○) or absence (■) of 1 mM unlabelled adenine. In the absence of inhibitor the transport rate was $3.9 \pm 0.2 \times 10^{-5}$ pmol $\cdot$ (10^7 cells)$^{-1} \cdot s^{-1}$ as calculated by linear regression ($r^2 = 0.98$). At 240 s, uptake was inhibited 70 % by 1 mM adenine. (**B**) Transport of 0.05 μM [^{3}H]adenine was inhibited by increasing concentrations of unlabelled adenine (○) or adenosine (■). Adenine uptake was expressed as a percentage of the control because two different experiments were combined, with no-inhibitor values of 2.4×10^{-4} and 1.5×10^{-4} pmol $\cdot$ (10^7 cells)$^{-1} \cdot s^{-1}$. Inset: conversion of the inhibition data into a Michaelis–Menten plot, yielding a K_m value of 0.12 μM for this experiment. Units of uptake were pmol $\cdot$ (10^7 cells)$^{-1} \cdot s^{-1}$. Values are means $\pm$ S.E.M.

and the fact that these experiments were carried out at 10 μM [^{3}H]adenine, which fully saturates PfADET1, as well as the low affinity for adenosine, clearly shows that this adenine transport phenomenon must be mediated by a low-affinity transporter separate from either PfNT1 or PfADET1. The low-affinity adenine transport activity was provisionally designated PfADET2.

Despite the presence of two adenine transporters, it is not at all clear whether adenine salvage is important to *Plasmodium* spp. and there is considerable controversy as to whether *Plasmodium* can utilize adenine at all, as several groups report the inability to identify genes encoding adenine phosphoribosyltransferase and methylthioadenosine phosphorylase in any *Plasmodium* species [35,36]; neither is there any evidence for the activity of adenine deaminase [35]. Yet, the characterization of adenine

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phosphoribosyltransferase from *P. chabaudi* and *P. falciparum* has been reported [37,38]. In addition, *P. falciparum* is reportedly able to grow *in vitro* on adenine as the sole purine source [39] and *P. knowlesi* incorporated adenine into nucleic acids [32]. Although these latter observations could be explained by conversion of the adenine into hypoxanthine in the host cell, Van Dyke [40] reported that the free parasite can also incorporate adenine into nucleic acids, albeit at 1 % of the efficiency by which hypoxanthine is incorporated. The results in the present study of uptake of adenine by *P. falciparum* trophozoites, however inefficient, would be compatible with the view that adenine can be utilized in some way but is unlikely to play any major role in the synthesis of nucleotides. It is conceivable that the parasite has a different use for low levels of adenine, which it cannot generate itself from other purines [35], or that adenine at high concentrations could be deaminated by adenosine deaminase.

Low-affinity transport of [^{3}H]adenosine in *P. falciparum* trophozoites

Several groups have reported a much lower-affinity adenosine transport process than the K_m value reported in the present study for PfNT1 [8,9,11]. We therefore decided to reinvestigate the presence of such a transporter and found that 25 μM [^{3}H]adenosine is taken up extremely rapidly at room temperature (see Supplementary Figure S3A at http://www.BiochemJ.org/bj/411/bj4110287add.htm), as reported by Downie et al. [11], reaching an accumulation ratio of $\sim$0.6 by 30 s. We therefore conducted our experiments at 6 °C and consistently found that uptake of 25 μM [^{3}H]adenosine was linear over a period of 4–6 s at this temperature (see Supplementary Figure S3B at http://www.BiochemJ.org/bj/411/bj4110287add.htm), and that initial rates of transport could therefore be assessed over a 3 s interval. At 22 °C, 10 °C or 6 °C we found that transport was saturable by 1 mM unlabelled adenosine, although this never inhibited fully 100 % of transport (Figure 4 and Supplementary Figure S3), reflecting the low affinity of this transporter for the substrate. At 6 °C the rate of uptake of 25 μM [^{3}H]adenosine was 0.041 ± 0.004 pmol $\cdot$ (10^7 cells)$^{-1}$. The mean K_m value under these conditions was 197 ± 20 μM, with a V_{max} of 0.19 ± 0.03 pmol $\cdot$ (10^7 cells)$^{-1} \cdot$ s^{-1} ($n = 3$; Figure 4). This transporter was not sensitive to the nucleoside transport inhibitor dipyridamole at concentrations of up to 25 μM (results not shown). We have designated this activity PfLAAT.

These observations are entirely consistent with the only other characterization of adenosine transport in *P. falciparum* trophozoites, by Downie et al. [11]. They reported a low-affinity adenosine transporter that mediated uptake of adenosine, inosine and thymidine very quickly, equilibrating within seconds, and insensitive to dipyridamole.

Disruption of the *PfNT1* gene

In order to gain insight into the possible role of PfNT1 in purine salvage, a parasite line with a disrupted locus was generated. Single crossover homologous recombination at the *PfNT1* locus was expected to generate a pseudodiploid configuration, with both truncated copies lacking some of the transmembrane domains of the protein predicted to be essential for the function of the protein (Supplementary Figure S4 at http://www.BiochemJ.org/bj/411/bj4110287add.htm). As the parasite is haploid and *PfNT1* is a single-copy gene, only one round of drug selection is necessary to obtain a null-mutant. Four parasite clones, denoted B11, D6, B7 and B9, were obtained, and successful disruption of the *PfNT1* gene was verified in each clone by PCR and by Southern blot (Supplementary Figures S5 and S6 at http://www.BiochemJ.org/bj/411/bj4110287add.htm). The mutant *PfNT1* clones were designated $\Delta pfnt1^{GU}$, in order to distinguish them from the independently derived $\Delta pfnt1$ clone of El Bissati et al. [17].

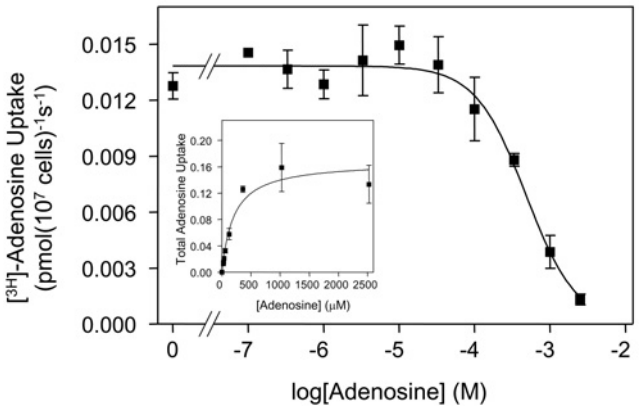

Figure 4 Low-affinity transport of adenosine in saponin-isolated *Plasmodium falciparum* trophozoites

Transport of 25 μM [^{3}H]adenosine was determined over a 3 s interval at 6 °C, in the presence of various concentrations of unlabelled adenosine as indicated. Inset: conversion into a Michaelis–Menten plot. Values are means $\pm$ S.E.M.

Purine transport in the $\Delta pfnt1^{GU}$ clone

We next determined the purine uptake profile in the $\Delta pfnt1^{GU}$ clone D6, using as controls untransformed parasites (wild-type) that had undergone the same procedures. The transport assays were performed simultaneously on both clones, to assess which, if any, of the above identified purine transport activities was affected in the parasites with the disrupted gene. Uptake of 25 μM [^{3}H]adenosine at 6 °C was not affected by the disruption of *PfNT1*, and identical rates of uptake were measured in the two lines: 0.069 ± 0.05 and 0.070 ± 0.07 pmol $\cdot$ (10^7 cells)$^{-1} \cdot$ s^{-1} for $\Delta pfnt1^{GU}$ and control respectively (Figure 5A).

In contrast, in several experiments, uptake of 0.25 μM [^{3}H]adenosine was reduced by 97–100 % in $\Delta pfnt1^{GU}$ ($n = 3$; Figure 5B), clearly indicating the loss of high-affinity transport in the mutant line. Similarly, uptake of 0.4 μM [^{3}H]hypoxanthine over 120 s was reduced by 82–98 % ($n = 3$; Figure 5C). The small residual hypoxanthine transport leads us to speculate that a low-affinity hypoxanthine activity might also be expressed in *P. falciparum*. This would be consistent with the observation of El Bissati et al. [17] that $\Delta pfnt1$ *P. falciparum* are able to grow in the presence of high concentrations of hypoxanthine but not in low concentrations.

Transport rates of 1 μM [^{3}H]adenine were not affected in the Δ*PfNT1* parasites: the initial rates of transport over 12 s were not significantly different in the $\Delta pfnt1^{GU}$ line and control cells ($n = 3$; paired Student's t test, $P > 0.05$) (Figure 5D and inset). However, in three independent experiments the maximum level of [^{3}H]adenine transport in the $\Delta pfnt1^{GU}$ line was double that of the control [0.16 ± 0.06 compared with 0.077 ± 0.022 pmol $\cdot$ (10^7 cells)$^{-1} \cdot$ s^{-1}; $n = 3$, non-linear regression]. Rather than up-regulation of the transporter we believe this may reflect an increased rate of adenine metabolism in the absence of a source for hypoxanthine.

The clear conclusion from these results is that *PfNT1* encodes the high-affinity hypoxanthine/adenosine transporter. This conclusion is somewhat at odds with the reports of PfNT1 as a relatively low-affinity transporter when expressed in

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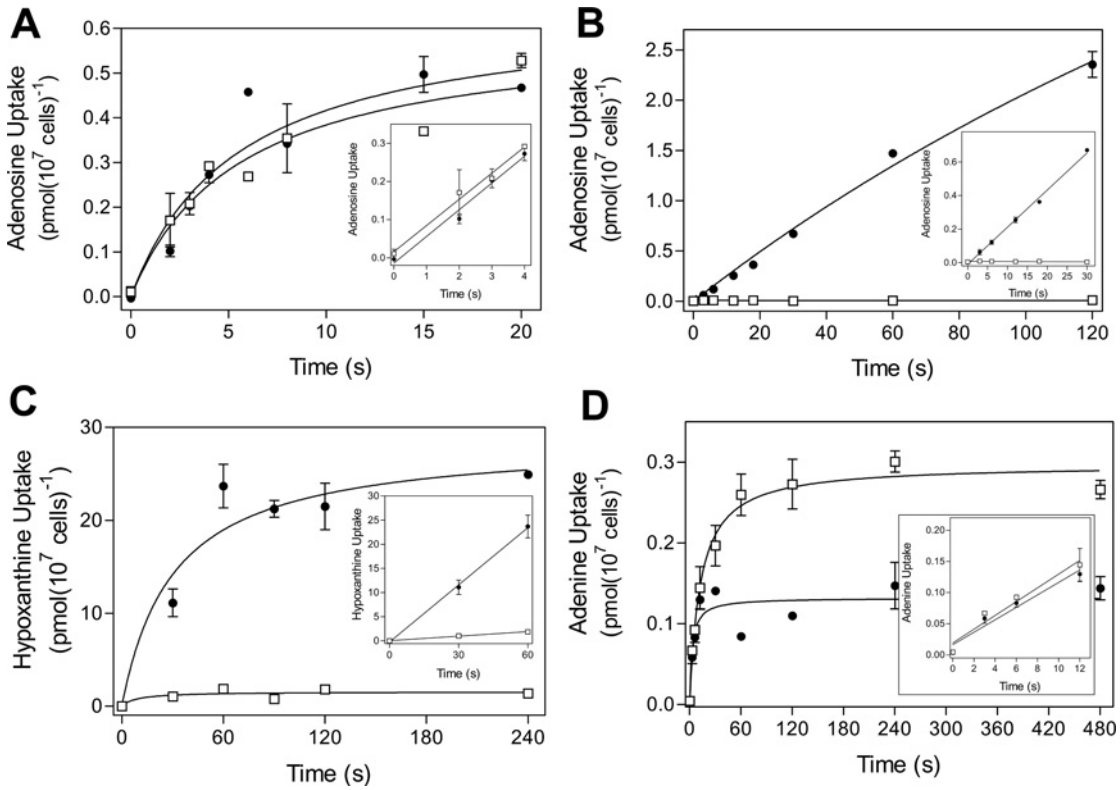

Figure 5 Purine transport in *P. falciparum* trophozoites lacking *PfNT1*

Transport in Δ*pfnt1*^{GU} is indicated with □ and in control 3D7 parasites with ●. (**A**) [^{3}H]Adenosine (25 μM) at 6 °C. (**B**) [^{3}H]Adenosine (0.25 μM) at 22 °C. (**C**) [^{3}H]Hypoxanthine (0.4 μM) at 22 °C. (**D**) [^{3}H]Adenine (1 μM) at 22 °C. Insets display the linear phase of uptake ($r^2 = 0.99$ for Δ*pfnt1*^{GU} and 0.98 for control 3D7).

Xenopus laevis oocytes [8,9,11]. However, these previous reports differ substantially from each other, producing adenosine K_m values of 320 μM, 13.2 μM and 1.86 mM respectively. It may be that the *Xenopus* expression system produces ambiguous results when expressing some *Plasmodium* transporters, possibly as a result of the extremely high A+T content of the *P. falciparum* genome [41] (*PfNT1* is 72 % A+T). This would also explain the reported difficulties of expressing other *P. falciparum* ENT genes in this system [11]. Also, the Carter et al. [9] study used a different parasite clone from the Parker et al. [8] and Downie et al. [11] studies (W2 and 3D7 respectively), which had a single amino acid difference in their *PfNT1* gene (leucine or phenylalanine respectively at position 385). This could perhaps explain some of the discrepancy, especially since the Phe^{385} polymorphism is unusual: in a multiple alignment of 28 protozoan ENT family genes plus the four human ENT sequences, the equivalent amino acid residue was conserved in so far as it was never aromatic, amide, charged, cysteine or proline. However, it is unlikely that the 3D7 clone of *P. falciparum* encodes a partly deficient *PfNT1*, as this clone was also used in the study reported by El Bissati et al. [17] that first suggested that PfNT1 is essential for growth on low purine concentrations. We also used the 3D7 clone in the present study and verified that its *PfNT1* sequence was 100 % identical with the entry in PlasmoDB (PF13_0252) and with the sequence reported by Parker et al. [8].

A new model for purine salvage by *P. falciparum*

The results of the present study, establishing a central role for a high-affinity hypoxanthine transporter in the purine salvage of *P. falciparum*, agree very well with the early studies of purine salvage in other *Plasmodium* species, which describe a transport system for hypoxanthine with a secondary capacity for purine nucleosides and a separate uptake system for adenine [1]. It is also consistent with the current understanding that purine nucleotide synthesis in *Plasmodium* spp. is overwhelmingly through phosphoribosylation of nucleobases, particularly hypoxanthine, rather than nucleoside kinases [4,5,42] and they do not seem to encode an adenosine kinase in their genome [35,36]. Furthermore, it brings purine uptake in *P. falciparum* very close to purine transport activities reported for other protozoa, which typically display K_m values in the 0.2–5 μM range for their substrates [1]. Earlier proposals that *P. falciparum* may rely exclusively on low-affinity purine transporters seemed hard to reconcile with a free concentration of purine nucleosides and bases in the infected erythrocyte that is presumably at best in the low micromolar range. Both the hENT1 nucleoside [43] and hFNT1 (human facilitative nucleoside transporter 1) nucleobase [27] transporters of human erythrocytes are equilibrative, making it impossible for the free concentrations of substrates to exceed those in plasma. Nor is it likely that purine concentrations are much higher at the parasite plasma membrane, as the parasitophorous vacuole membrane is freely permeable to such low-molecular-mass solutes [30].

However, at adenosine concentrations that saturate the high-affinity PfNT1 transporter, a very rapid and saturable nucleoside transport activity, PfLAAT, was observed. Our transport experiments with Δ*pfnt1*^{GU} prove that this is indeed a separate transporter from PfNT1. We conclude that *P. falciparum* trophozoites express both a high-affinity purine nucleobase/nucleoside transporter and a low-affinity/high-capacity adenosine

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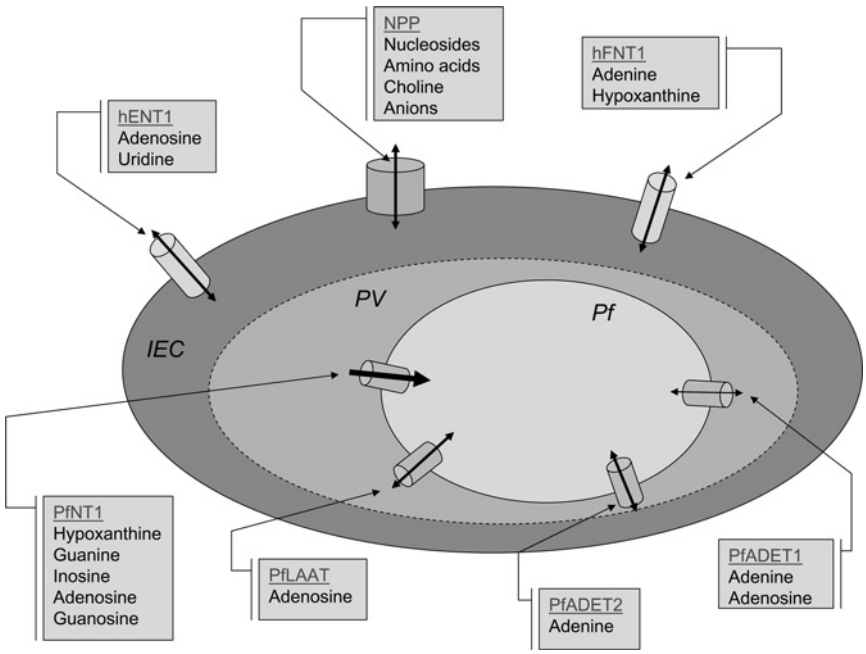

Figure 6 Model of purine uptake into intraerythrocytic *P. falciparum* trophozoites

Double-headed arrows indicate presumed equilibrative transport, whereas the single-headed arrow of PfNT1 indicates possible active transport. However, the assignment of active or equilibrative transport is speculative and was not investigated in the present study. hFNT1 and hENT1 are endogenous to the human erythrocyte whereas the origin of the NPP (new permeation pathways) is unknown. The thickness of arrows for the *P. falciparum* transporters is meant to convey the relative flux of purines. IEC, infected erythrocyte cytoplasm; PV, parasitophorous vacuole.

transporter in addition to a saturable transport system for [^{3}H]adenine. The K_m value of $\sim$200 μM for PfLAAT rules out the possibility that it might be identical with the high-affinity adenine transporter PfADET1 [K_i(adenosine) = 2.0 μM] and equally appears to rule out that it is identical with the low-affinity adenine transport activity PfADET2 that is almost insensitive to adenosine. However, this awaits proof, consisting of experiments with genetic deletion lines of the genes encoding these purine transporters (which are currently in progress).

Thus a model arises (shown in Figure 6) that closely mirrors the model for purine transport in the only other apicomplexan parasite for which purine transport has been studied in detail. *Toxoplasma gondii* is known to express a low-affinity adenosine transporter TgAT1, with a K_m value of $\sim$110 μM [13,44,45], as well as a high-affinity transporter TgAT2 (K_m is 0.49 μM for adenosine and 0.77 μM for inosine) [13]. The main difference between purine salvage in the two apicomplexan species is that, unlike *P. falciparum, T. gondii* expresses separate high-affinity transporters for purine nucleosides (TgAT2) and oxopurine nucleobases (TgNBT1). Like PfNT1, TgNBT1 does not transport adenine [13].

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REFERENCES

1 De Koning, H. P., Bridges, D. J. and Burchmore, R. (2005) Purine and pyrimidine transport in pathogenic protozoa: from biology to therapy. FEMS Microbiol. Rev. **29**, 987–1020

2 Tracy, S. M. and Sherman, I. W. (1972) Purine uptake and utilization by the avian malaria parasite *Plasmodium lophurae*. J. Protozool. **19**, 541–549

3 Hansen, B. D., Sleeman, H. and Pappas, P. W. (1980) Purine base and nucleoside uptake in *Plasmodium berghei* and host erythrocytes. J. Parasitol. **66**, 205–212

4 Reyes, P., Rathod, P. K., Sanchez, D. J., Mrema, J. E. K., Rieckmann, K. H. and Heidrich, H. G. (1982) Enzymes of purine and pyrimidine metabolism from the human malaria parasite *Plasmodium falciparum*. Mol. Biochem. Parasitol. **5**, 275–290

5 Manandhar, M. S. P. and Van Dyke, K. (1975) Detailed purine salvage metabolism in and outside the free malarial parasite. Exp. Parasitol. **37**, 138–146

6 Berman, P. A., Human, L. and Freese, J. A. (1991) Xanthine oxidase inhibits growth of *Plasmodium falciparum* in human erythrocytes *in vitro*. J. Clin. Invest. **88**, 1848–1855

7 Penny, J. I., Hall, S. T., Woodrow, C. J., Cowan, G. M., Gero, A. M. and Krishna, S. (1998) Expression of substrate-specific transporters encoded by *Plasmodium falciparum* in *Xenopus laevis* oocytes. Mol. Biochem. Parasitol. **93**, 81–89

8 Parker, M. D., Hyde, R. J., Yao, S. Y. M., McRobert, L., Cass, C. E., Young, J. D., McConkey, G. A. and Baldwin, S. A. (2000) Identification of a nucleoside/nucleobase transporter from *Plasmodium falciparum*, a novel target for antimalarial chemotherapy. Biochem. J. **349**, 67–75

9 Carter, N. S., Ben Mamoun, C., Liu, W., Silva, E. O., Landfear, S. M., Goldberg, D. E. and Ullman, B. (2000) Isolation and functional characterization of the PfNT1 nucleoside transporter gene from *Plasmodium falciparum*. J. Biol. Chem. **275**, 10683–10691

10 Rager, N., Ben Mamoun, C., Carter, N. S., Goldberg, D. E. and Ullman, B. (2001) Localization of the *Plasmodium falciparum* PfNT1 nucleoside transporter to the parasite plasma membrane. J. Biol. Chem. **276**, 41095–41099

11 Downie, M. J., Saliba, K. J., Howitt, S. M., Bröer, S. and Kirk, K. (2006) Transport of nucleosides across the *Plasmodium falciparum* parasite plasma membrane has characteristics of PfENT1. Mol. Microbiol. **60**, 738–748

12 Ghosh, M. and Mukherjee, T. (2000) Stage-specific development of a novel adenosine transporter in *Leishmania donovani* amastigotes. Mol. Biochem. Parasitol. **108**, 93–99

13 De Koning, H. P., Al-Salabi, M. I., Cohen, A., Coombs, G. and Wastling, J. M. (2003) Identification and characterization of high affinity purine nucleoside and nucleobase transporters in *Toxoplasma gondii*. Int. J. Parasitol. **33**, 821–831

14 Al-Salabi, M. I. and De Koning, H. P. (2005) Purine nucleobase transport in amastigotes of *Leishmania mexicana*: involvement in allopurinol uptake. Antimicrob. Ag. Chemother. **49**, 3682–3689

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15 Carter, N. S. and Fairlamb, A. H. (1993) Arsenical-resistant trypanosomes lack an unusual adenosine transporter. Nature **361**, 173–176
16 De Koning, H. P. and Jarvis, S. M. (1997) Purine nucleobase transport in bloodstream forms of *Trypanosoma brucei* is mediated by two novel transporters. Mol. Biochem. Parasitol. **89**, 245–258
17 El Bissati, K., Zufferey, R., Wittola, W. H., Carter, N. S., Ullman, B. and Ben Mamoun, C. (2006) The plasma membrane permease PfNT1 is essential for purine salvage in the human malaria parasite *Plasmodium falciparum*. Proc. Natl. Acad. Sci. U.S.A. **103**, 9286–9291
18 Martin, R. E., Henry, R. I., Abbey, J. L., Clements, J. D. and Kirk, K. (2005) The 'permeome' of the malaria parasite: an overview of the membrane transport proteins of *Plasmodium falciparum*. Genome Biol. **6**, R26
19 Trager, W. and Jensen, J. B. (1976) Human malaria parasites in continuous culture. Science **193**, 673–675
20 Lambros, C. and Vanderberg, J. P. (1979) Synchronization of *Plasmodium falciparum* erythrocytic stages in culture. J. Parasitol. **65**, 418–420
21 Stead, A. M. W., Bray, P. G., De Koning, H. P., Elford, B. C., Stocks, P. A. and Ward, S. A. (2001) Diamidine compounds: selective uptake and targeting in *Plasmodium falciparum*. Mol. Pharmacol. **59**, 1298–1306
22 Beaumelle, B. D., Vial, H. J. and Phillippot, J. R. (1987) Reevaluation, using marker enzymes, of the ability of saponin and ammonium chloride to free *Plasmodium* from infected erythrocytes. J. Parasitol. **73**, 743–748.
23 Saliba, K. J. and Kirk, K. (1999) pH regulation in the intracellular malaria parasite, *Plasmodium falciparum*. H^+ extrusion via H^+-ATPase. J. Biol. Chem. **274**, 33213–33219
24 Allen, R. J. W. and Kirk, K. (2004) The membrane potential of the intraerythrocytic malaria parasite *Plasmodium falciparum*. J. Biol. Chem. **279**, 11264–11272
25 De Koning, H. P., Watson, C. J. and Jarvis, S. M. (1998) Characterisation of a nucleoside/proton symporter in procyclic *Trypanosoma brucei brucei*. J. Biol. Chem. **273**, 9486–9494
26 Al-Salabi, M. I., Wallace, L. J. M. and De Koning, H. P. (2003) A *Leishmania major* nucleobase transporter responsible for allopurinol uptake is a functional homologue of the *Trypanosoma brucei* H2 transporter. Mol. Pharmacol. **63**, 814–820
27 Wallace, L. J. M., Candlish, D. and De Koning, H. P. (2002) Different substrate recognition motifs of human and trypanosome nucleobase transporters: selective uptake of purine antimetabolites. J. Biol. Chem. **277**, 26149–26156
28 Saliba, K. J., Horner, H. A. and Kirk, K. (1998) Transport and metabolism of the essential vitamin pantothenic acid in human erythrocytes infected with the malaria parasite *Plasmodium falciparum*. J. Biol. Chem. **273**, 10190–10195
29 Sidhu, A. B., Valderramos, S. G. and Fidock, D. A. (2005) Pfmdr1 mutations contribute to quinine resistance and enhance mefloquine and artemisinin sensitivity in *Plasmodium falciparum*. Mol. Microbiol. **57**, 913–926
30 Desai, S. A., Krogstad, D. J. and McCleskey, E. W. (1993) A nutrient-permeable channel on the intraerythrocytic malaria parasite. Nature **362**, 643–646
31 Stein, A., Vaseduvan, G., Carter, N. S., Ullman, B., Landfear, S. M. and Kavanaugh, M. P. (2003) Equilibrative nucleoside transporter family members from *Leishmania*. J. Biol. Chem. **278**, 35127–35134
32 Gutteridge, W. E. and Trigg, P. I. (1970) Incorporation of radioactive precursors into DNA and RNA of *Plasmodium knowlesi in vitro*. J. Protozool. **17**, 89–96
33 Too, K., Brown, D. M., Bongard, E., Yardley, V., Vivas, L. and Loakes, D. (2007) Anti-malarial activity of N6-modified purine analogs. Bioorg. Med. Chem. **15**, 5551–5562
34 Goudela, S., Karatza, P., Koukaki, M., Frilingos, S. and Diallinas, G. (2005) Comparative substrate recognition by bacterial and fungal purine transporters of the NAT/NCS2 family. Mol. Membr. Biol. **22**, 263–275
35 Ting, L. M., Shi, W., Lewandowicz, A., Singh, V., Mwakingwe, A., Birck, M. R., Ringia, E. A., Bench, G., Madrid, D. C., Tyler, P. C. et al. (2005) Targeting a novel *Plasmodium falciparum* purine recycling pathway with specific immucillins. J. Biol. Chem. **280**, 9547–9554
36 Chaudhary, K., Darling, J. A., Fohl, L. M., Sullivan, W. J., Donald, R. G., Pfefferkorn, E. R., Ullman, B. and Roos, D. S. (2004) Purine salvage pathways in the apicomplexan parasite *Toxoplasma gondii*. J. Biol. Chem. **279**, 31221–31227
37 Walter, R. D. and Königk, E. (1974) Hypoxanthine-guanine phosphoribosyltransferase and adenine phosphoribosyltransferase from *Plasmodium chabaudi*; purification and properties. Tropenmed. Parasitol. **25**, 227–235
38 Queen, S. A., Vander Jagt, D. L. and Reyes, P. (1989) Characterisation of adenine phosphoribosyltransferase from the human malaria parasite, *Plasmodium falciparum*. Biochim. Biophys. Acta **996**, 160–165
39 Geary, T. G., Divo, A. A., Bonanni, L. C. and Jensen, J. B. (1985) Nutritional requirements of *Plasmodium falciparum* in culture. III. Further observations on essential nutrients and antimetabolites. J. Protozool. **32**, 608–613
40 Van Dyke, K. (1975) Comparison of tritiated hypoxanthine, adenine and adenosine for purine salvage incorporation into nucleic acids of the malarial parasite, *Plasmodium falciparum*. Tropenmed. Parasitol. **26**, 232–238
41 Gardner, M. J., Hall, N., Fung, E., White, O., Berriman, M., Hyman, R. W., Carlton, J. M., Pain, A., Nelson, K. E., Bowman, S. et al. (2002) Genome sequence of the human malaria parasite *Plasmodium falciparum*. Nature **419**, 498–511
42 Berens, R. L., Krug, E. C. and Marr, J. J. (1995) Purine and pyrimidine metabolism. In Biochemistry and Molecular Biology of Parasites (Marr, J. J. and Müller, M., eds.), pp. 89–117, New York, Academic Press
43 Plagemann, P. G. W., Wohlhueter, R. M. and Woffendin, C. (1988) Nucleoside and nucleobase transport in animal cells. Biochim. Biophys. Acta **947**, 405–443
44 Schwab, J. C., Afifi Afifi, M., Pizzorno, G., Handschumacher, R. E. and Joiner, K. A. (1995) *Toxoplasma gondii* tachyzoites possess an unusual plasma membrane adenosine transporter. Mol. Biochem. Parasitol. **70**, 59–69
45 Chiang, C.-W., Carter, N., Sullivan, W. J., Donald, R. G., Roos, D. S., Naguib, F. N., El Kouni, M. H., Ullman, B. and Wilson, C. M. (1999) The adenosine transporter of *Toxoplasma gondii*; identification by insertional mutagenesis, cloning, and recombinant expression. J. Biol. Chem. **274**, 35255–35261

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